Scientific Report

Project: Engineered glycopeptide-based micro/nanomotors for anti-tumoral co-drug release
Acronym: GlyPepTum
Code: PN-III-P1-1.1-PD-2019-0271, **No**. 149/2020

Stage no. 2 / 2021

Preparation and characterization of magnetic nanoparticles conjugated with catalytic enzymes for self-propulsion and guidance of the micro/nanorobots. Design and synthesis of new polymersomes with a stomatocyte shape - preliminary studies

- **Summary of the scientific report**

In the second stage, according to the project plan, the preparation and characterization of magnetic nanoparticles conjugated with enzymes with catalytic activity (glucose oxidase) for self-propulsion and guidance of micro / nanorobots were performed as well as preliminary studies on the design and synthesis of new stomatocyte-shaped polymersomes.
Magnetic nanoparticles (NMPs) are widely used as a support for immobilizing biomolecules by physical or chemical methods to obtain a high degree of loading. However, simple magnetic particles have low colloidal stability, especially at neutral pH, and therefore need stabilization and surface modification.
Glucose oxidase (GOx) has low stability and it is not possible to use this enzyme in several catalytic cycles. One of the most promising ways to improve the disadvantages of this enzyme is immobilization. The immobilization strategy has a great impact on the properties of the attached enzymes, being able to improve both the activity, the stability and the selectivity, but also the specificity of the enzyme.
Although in the case of immobilization of enzymes on the surface of magnetic nanoparticles, the methods of coating nanoparticles with natural or synthetic polymers are preferred, in the absence of polymeric coating materials, the nanoparticle size does not increase and therefore the high coupling capacity and magnetic properties will remain unaffected. It has been reported in the literature that in the case of large magnetic aggregates coated with polymers there occur also the nonspecific uptake of some compounds contained in the immobilization medium, and in the case of smaller uncoated magnetic nanoparticles the nonspecific binding is reduced. Thus, uncovered magnetic nanoparticles are a more suitable support for immobilizing enzymes, while providing a simple method of preparation that also involves low costs.
At this stage, GOx-coated magnetic nanoparticles were obtained by forming Schiff bases between the aldehyde groups introduced on the surface of the magnetic nanoparticles and the terminal free amino groups of the enzyme. Two bifunctional compounds were used for the functionalization of the particles: glutaraldehyde and squaric acid, a compound with low cytotoxicity, and their properties in relation to the degree of immobilization of the enzyme and the stability in buffers that simulate the biological

environment were compared, obtaining the variant of GOx magnetic nanoparticles to be used in the formation of hybrid polymersomes.

Synthesis of magnetic nanoparticles conjugated with catalytic enzymes

Fe_3O_4 magnetic nanoparticles were synthesized using the conventional co-precipitation method, described in the literature. Due to the presence of hydroxyl groups on the surface of magnetic nanoparticles (MNP) that are able to react with the oxyethyl side chain of (3-aminopropyl) triethoxysilanes (APTES), the surface of MNP was modified. The APTES-modified magnetic nanoparticles (AMNP) were reacted with two bifunctional compounds with aldehyde groups: glutaric aldehyde (GA) and squaric acid (SA).

Improvement of enzyme immobilization protocol on magnetic nanoparticles

In the case of magnetic nanoparticles functionalized with SA, by increasing the enzyme ratio there was an increase in the potential value from -29.9 mV for AMNP_SA0.5_GOx16/1 to -25.6 mV for AMNP_SA0.5_GOx4/1, but also a decrease in size particles and PDI, which indicates an increased stability of magnetic particles functionalized with SA and conjugated with GOx due to the combined steric and electrostatic effects. In contrast, for GOx-conjugated GA-functionalized magnetic nanoparticles only at the nanoparticle / enzyme ratio of 4/1 g /g, a good stability was observed compared to the other ratios used (8/1 and 16/1, respectively).

The increase in immobilization time led to an increase in the stability of the particles functionalized with both GA and SA due to the increase in the amount of enzyme immobilized on the particles.

Characterization of enzyme-conjugated magnetic nanoparticles

Structural analysis by FT-IR spectroscopy confirmed the various steps of GOx coupling on the surface of functionalized magnetic nanoparticles.

According to SEM images, most particles were in the form of agglomerated structures due to their magnetic properties, and GOx-conjugated nanoparticles had a cluster-like conformation due to the coupling of biological macromolecules.

The magnetic properties were characterized by recording the graphs corresponding to the saturation magnetization (Ms). A gradual decrease in Ms was observed for GOx-conjugated magnetic nanoparticles. This decrease in Ms could be attributed to APTES functionalization and enzyme coupling on the MNP surface.

Due to the high stability of SA-functionalized magnetic nanoparticles compared to GA-functionalized ones, SA-functionalized and GOx-coated magnetic nanoparticles were characterized in terms of thermal stability, immobilization, and residual enzymatic activity.

In the case of nanoparticles AMNP_SA0.5_GOx4/1= 3:1, the second stage of the thermal degradation process was attributed to the degradation of the enzyme, the release of carboxyl and amino groups contained in GOx.

Assessment of enzyme-conjugated nanoparticle activity and stability in simulated biological media

The optimal immobilization time of the enzyme on the MNP was 8 hours.
Immobilized enzyme activity was: 0.008, 0.032 and 0.1 U / mg AMNP_SA0.5 for 16: 1, 8: 1 and 4: 1 ratios. By increasing the amount of SA-functionalized magnetic nanoparticles, the amount of immobilized enzyme was lower, indicating that the main limitations may be the concentration of the enzyme or that there is a competition for coupling with other residual proteins remaining from the obtainment of GOx.

Design and synthesis of new polymersomes with stomatocyte shape – preliminary studies
Glycopeptide-based polymersomes were obtained by self-assembly using the nanoprecipitation method by dialysis, and the particle size obtained by nanoprecipitation was predetermined by the molecular composition (ratio of peptide segments - leucine and CBZ-lysine) and glycopeptide concentration.
Preliminary tests for encapsulation of magnetic particles in polymersomes based on MAC-pLeu were performed at the MAC-pLeu ratio: AMNP_SA0.5_GOx4 / 1, and their morphology was characterized by recording microscopic images (TEM).

<u>Dissemination</u>
Scientific results – Research Articles:

- Submission for publication of an article in ISI journals or dedicated journals or in open access / green open access journals: *Alina Gabriela Rusu, Aurica P. Chiriac, Loredana Elena Nita, Alina Ghilan, Daniela Rusu, Natalia Simionescc, Liliana Mititelu Tartau. Nanostructured hyaluronic acid-based hydrogels encapsulating synthetic/ natural hybrid nanogels as promising wound dressings. Biochemical Engineering Journal,* ***IF 3.978*** *(under review)*

Scientific results – International Conferences:

- participation at international conferences with poster presentations:
    1. *Synthesis and characterization of maleoyl-chitosan/polypeptide bioconjugate; Alina Gabriela Rusu, Aurica P. Chiriac, Loredana Elena Nita; 7th International Polysaccharide Conference (EPNOE 2021), Nantes, France, 11-15 October 2021 (poster).*
    2. *Enzymatic crosslinked hydrogels based on gelatin loaded with maleoyl-chitosan/poly (aspartic acid) nanocarriers as potential therapeutic scaffolds for tissue engineering, Alina Gabriela Rusu, Aurica P. Chiriac, Loredana Elena Nita, 31st Annual Conference of the European Society for Biomaterials (ESB 2021), Porto, Portugal, fully virtual, 5-9th September 2021 (poster).*